TGF-β induces assembly of a Smad2–Smurf2 ubiquitin ligase complex that targets SnoN for degradation

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The receptor-regulated Smad proteins are essential intracellular mediators of signal transduction by the transforming growth factor-β (TGF-β) superfamily of growth factors and are also important as regulators of gene transcription. Here we describe a new role for TGF-β-regulated Smad2 and Smad3 as components of a ubiquitin ligase complex. We show that in the presence of TGF-β signalling, Smad2 interacts through its proline-rich PPXY motif with the tryptophan-rich WW domains of Smurf2, a recently identified E3 ubiquitin ligases. TGF-β also induces the association of Smurf2 with the transcriptional co-repressor SnoN and we show that Smad2 can function to mediate this interaction. This allows Smurf2 HECT domain to target SnoN for ubiquitin-mediated degradation by the proteasome. Thus, stimulation by TGF-β can induce the assembly of a Smad2–Smurf2 ubiquitin ligase complex that functions to target substrates for degradation.

The pleiotropic TGF-β superfamily of cytokines signal through transmembrane Ser/Thr kinase receptors[1–3]. Upon ligand binding, a heteromeric complex forms between the type II and type I receptors in which the type II kinase transphosphorylates a series of serines residues in the glycine- and serine-rich (GS) region of the type I receptor. This induces the type I receptor to activate downstream intracellular signal transducers of the Smad family of proteins[2,4–7]. The receptor-regulated Smads (R-Smads) are targeted and phosphorylated by the activated type I receptor on a C-terminal SSXS motif in a selective manner[8–11]. Specifically, R-Smad2 and 3 are recognized by the TGF-β/activin type I receptors, whereas R-Smads1, 5 and 8 are regulated by bone morphogenetic protein (BMP) type I receptors. Phosphorylated R-Smads can then form a heteromeric complex with the common partner Smad4 and the complex accumulates in the nucleus. Once in the nucleus, the complex can bind to specific promoter elements through interactions between the R-Smads and specific DNA-binding partners[12]. DNA-bound Smad complexes then regulate transcription, either positively through recruitment of coactivators such as the histone acetyltransferase CBP/p300 (refs 4–6, 12) or negatively by recruiting co-repressors such as 5′-TG-3′-interacting factor (TGIF)[13] and the Ski/SnoN family[14–19]. In addition to the R-Smads and Smad4, the inhibitory Smads (I-Smads) Smad6 and 7 disrupt TGF-β and BMP signalling through interactions with the activated receptors[20–22].

Proteolytic degradation of intracellular proteins by the ubiquitin-proteasome pathway is essential for diverse processes such as signal transduction, cell-cycle progression and endocytosis[23–25]. Ubiquitination is a posttranslational modification that involves the covalent attachment of chains of ubiquitin, a highly conserved 76-amino-acid protein, to target proteins. This process is achieved by the concerted actions of three different enzymes, the E1 ubiquitin activating enzyme, the E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. The principal role of E3 ubiquitin ligases is in determining substrate specificity[24,26]. Accordingly, several classes of these ubiquitin ligases have been defined. One of these classes, typified by the Skp–Cdc53/Cullin–F-box receptor (SCF) complex, consists of multicomponent complexes that act by transferring ubiquitin directly from the E2 to associated substrates[24,25]. Another major class of ubiquitin ligases contains HECT domains, which can catalyse the addition of ubiquitin to substrates[24,26]. Within the HECT family of E3s, a subfamily has been identified, C2-WW-HECT, which is characterized by a calcium-dependent phospholipid-binding domain (or C2 domain) at the amino-terminus, followed by two to four WW domains in the middle of the protein and the HECT domain at the carboxy terminus[27]. The WW domains are protein–protein-interaction domains that recognize proline-rich PPXY (PY) motifs[28]. Smurf1, a member of the C2-WW-HECT ubiquitin-ligase family, is an antagonist of the BMP signalling pathway[29]. Smurf1 can interact with the PY motifs of R-Smads 1 and 5 and can mediate ubiquitination and proteasomal degradation of these Smads. Smurf2, a recently identified Smurf1-related ligase, can also target Smad1 for degradation and may have some activity towards Smad2[30,31]. Furthermore, Smurf2 binds to Smad7 (ref. 32). Smad7 does not seem to be targeted for degradation by Smurf2 but rather functions to recruit Smurf2 to TGF-β receptor complexes from which Smurf2 then induces ubiquitin-dependent downregulation of the receptor–Smad7 complex. These results indicate that C2-WW-HECT ubiquitin ligases can be targeted to their substrates by adaptor proteins. As other Smads possess PY motifs, the role of Smad7 as a component of a ubiquitin ligase complex might also apply to other Smads.

The related nuclear proteins Ski and SnoN are transcriptional co-repressors[33] that have been reported to associate with the TGF-β-regulated Smads, Smad2 and 3, and their common partner, Smad4. This association leads to the recruitment of the N-CoR and histone deacetylase (HDAC) transcriptional co-repressor complex to specific promoters and causes repression of TGF-β responsive genes[14–19]. To antagonize this effect, TGF-β-stimulation of cells in turn leads to an increase in the turnover of SnoN in a proteasome-dependent

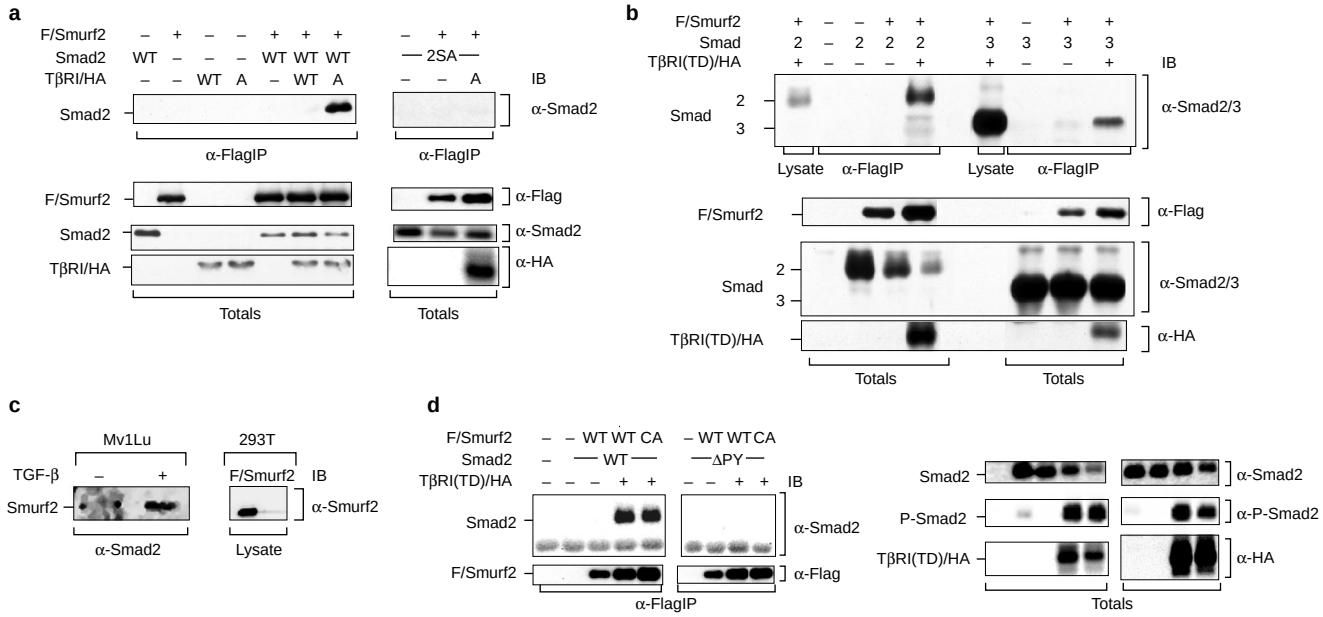

Figure 1 **TGF-β signalling induces association between Smurf2 and Smad2 or Smad3. a**, **b**, Interaction between F/Smurf2 and Smad2 or Smad3. 293T cells were transfected with the indicated combinations of Flag-tagged Smurf2 (F/Smurf2), untagged wild-type (WT) or a phosphorylation mutant (2SA) of Smad2, or untagged Smad3, together with wild-type (WT) or a constitutively active form (A) of HA-tagged TGF-β type I receptor (TβRI/HA). Cell lysates were subjected to Smurf2 immunoprecipitation (IP) and immmunoblotting (IB) with anti-Smad2/3 antibodies. Protein expression levels of F/Smurf2, Smad2, Smad3 and TβRI/HA were confirmed by immunoblotting with the appropriate antibodies as shown in the lower panels. **c**, TGF-β-dependent interaction between endogenous Smad2/3 and Smurf2. Lysates of untreated or TGF-β-treated Mv1Lu cells were subjected to a Smad2/3 immunoprecipitation, and endogenous co-immunoprecipitating Smurf2 was detected by immunoblotting with an antisera to Smurf2 (left panel). Lysates of 293T cells transfected with Flag-tagged Smurf2 were immunoblotted with Smurf2 antibody as a positive control (right panel). **d**, Smad2(ΔPY) does not interact with Smurf2. 293T cells were transiently transfected with different combinations of Smad2 (WT or ΔPY mutant in which the amino-acid residues 221–225 spanning the PY motif were deleted), F/Smurf2 (WT or Smurf2(C716A) HECT-domain mutant (CA)) in the presence or absence of activated TβRI/HA (TβRI(TD)/HA). Smurf2 was immunoprecipitated from lysates and co-immunoprecipitated Smad2 was detected by immunoblotting (upper left panels). Receptor-induced phosphorylation of Smad2 was determined by immunoblotting cell lysates with an anti-phospho-Smad2-specific (α-P-Smad2) antibody (middle right panels).

manner[16,18]. The mechanism by which SnoN is degraded by the TGF-β pathway is not known. Here we describe a new function for R-Smad2 and R-Smad3 as components of a ubiquitin-ligase. We show that Smad2 forms a complex with Smurf2 in a TGF-β-dependent manner, and that Smad2 can recruit Smurf2 to SnoN, thus targeting SnoN for degradation by the proteasome pathway.

Results

Smurf2 forms a complex with phosphorylated Smad2 but does not target it for degradation. The E3 ubiquitin ligase Smurf2 interacts constitutively with Smad7 through the Smad7 PY motif, and TGF-β activation results in Smad7-dependent recruitment of Smurf2 to TGF-β receptors[32]. Smurf2 in turn promotes the degradation of the Smad7–TGF-β receptor complex through the proteasomes and lysosomes. In these studies[32], we found that unlike Smad7, the PY-containing R-Smads did not interact with Smurf2 in unactivated cells. Thus, we asked whether activation of R-Smads by the type I receptor might regulate their interactions with Smurf2. For this, we focused on the TGF-β-regulated Smads, Smad2 and Smad3. Consistent with our previous findings[32], Smad2 did not coprecipitate with Smurf2 in unstimulated cells (Fig. 1a). However, in the presence of constitutively activated TGF-β type I receptor (TβRI), Smad2 associated with Smurf2, and a phosphorylation mutant of Smad2, Smad2(2SA)[10,11], which lacks the receptor-dependent phosphorylation sites, was unable to associate with Smurf2 (Fig. 1a). A similar preference of Smurf2 for binding phosphorylated Smad2 has also been reported[30]. In contrast, Smurf2 did not interact with Smad4, which lacks a PY motif, either in the absence or presence of activated TβRI receptor (data not shown). Similar to Smad2, the other TGF-β-regulated R-Smad, Smad3, interacted with Smurf2 in a TGF-β-dependent manner (Fig. 1b). To confirm that TGF-β signalling induced association of phosphorylated Smad2 and Smad3 with Smurf2, we investigated the association of these two proteins at endogenous levels of expression using Mv1Lu cells, which express Smurf2. In the absence of stimulation by TGF-β, Smurf2 did not coprecipitate with Smad2/3 (Fig. 1c). However, in cells stimulated with TGF-β, endogenous Smurf2 co-immunoprecipitated with Smad2/3 protein. Thus, TGF-β induces formation of a Smad2–Smurf2 complex. Smurf2 resides predominantly in the nucleus in certain cells[32], and TGF-β-dependent phosphorylation induces accumulation of Smad2 in the nucleus[3,7]. Therefore, colocalization of Smurf2 and activated Smad2/3 in the nucleus may explain the requirement for TGF-β signalling for this interaction. However, *in vitro* binding studies showed that Smurf2 retained specificity for phosphorylated Smad2 (data not shown). Together these data show that Smurf2 interacts stably with phosphorylated Smad2 and Smad3 in response to TGF-β signalling.

The association of C2-WW-HECT ubiquitin ligases with adaptors and substrates is mediated through interactions between the WW domains and PY motifs[27,23]. We therefore explored the role of the Smad2 PY motif in mediating binding to Smurf2. For this, we tested the interaction between Smurf2 and a mutant of Smad2, Smad2(ΔPY), which lacks the amino-acid residues 221–225 spanning the PY motif. As opposed to wild-type Smad2, which interacted strongly with Smurf2 in the presence of TGF-β signalling,

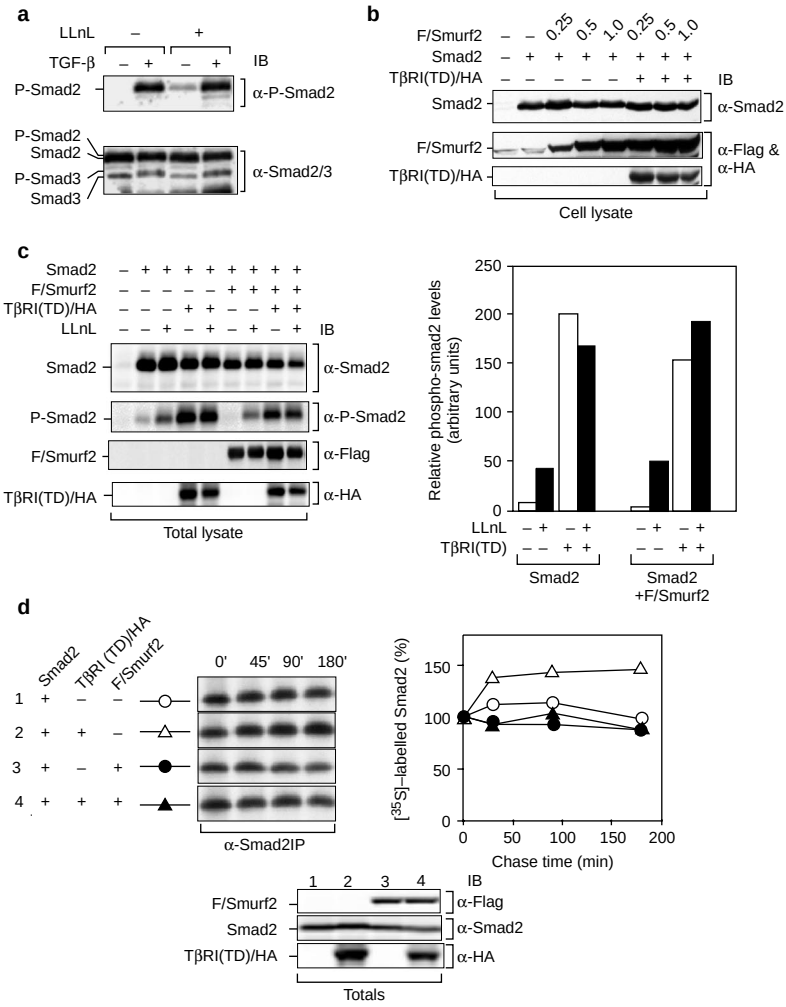

Figure 2 **Smad2 is not a major target for Smurf2-dependent degradation. a**, Endogenous levels of Smad2 and Smad3 at steady state are not altered significantly by proteasome inhibitors. Lysates of U4A/Jak1 cells, pretreated with the proteasomal inhibitor LLnL (40 μM) or a vehicle for 4 h, and incubated in the absence or presence of TGF-β (500 pM) for 30 min, were subjected to Smad2/3 immunoprecipitation. Endogenous phosphorylated Smad2 concentrations in immunoprecipitates of Smad2 were determined by immunoblotting with an anti-phospho-Smad2 specific antibody (α-P-Smad2; upper panel) and total Smad2 and 3 concentrations were determined by blotting with an anti-Smad2/3 antibody (lower panel). **b**, 293T cells were transfected with either Smad2 alone or Smad2 together with increasing concentrations of Smurf2 in the presence or absence of activated TβRI/HA and aliquots of cell lysates immunoblotted to assess steady-state concentrations of Smad2. The expression of Flag-tagged Smurf2 and TβRI(TD)/HA were confirmed by immunoblotting total cell lysates. **c**, Smurf2 does not alter the ratio of phosphorylated Smad2 relative to total Smad2, in the presence or absence of proteasome inhibitors. 293T cells were transfected as indicated, and incubated overnight with or without LLnL. The steady-state level of total and phosphorylated Smad2 in the cell lysates were visualized by immunoblotting (left panel), quantified using a BioRad Fluor-S-Multimager and the data were plotted as the ratio of phosphorylated Smad2 to total Smad2 (right panel). **d**, Smurf2 does not alter the rate of degradation of Smad2. 293T cells transiently transfected with the indicated expression constructs were pulse-labelled for 10 min with [^{35}S]-methionine and chased for the indicated times in media containing unlabelled methionine. [^{35}S]-Smad2 was immunoprecipitated (upper panels) and quantified by phosphorimage analysis. Labelled Smad2 is plotted at each time point as the percentage of amount present at time 0. The data represent the average of two independent experiments. Aliquots of total cell lysates were immunoblotted to confirm expression of F/Smurf2, Smad2 and TβRI(TD)/HA (lower panels).

Smad2(ΔPY) did not associate with Smurf2 (Fig. 1d, left panels). The loss of interaction between Smurf2 and Smad2(ΔPY) was not due to lack of phosphorylation by the receptor, as this mutant was phosphorylated to the same degree as wild-type Smad2 (Fig. 1d). Thus, the PY motif in Smad2 is critical for its interaction with Smurf2.

As Smurf2 is a ubiquitin ligase that interacts with phosphorylated Smad2, we next asked whether Smurf2 targets Smad2 for degradation. First, we investigated whether endogenous Smad2 is subject to proteasome-dependent degradation in cells that express endogenous Smurf2 (Fig. 2a). Analysis of Smad2 and phosphorylated Smad2, either in the presence or absence of TGF-β, revealed little proteasome-dependent change in total Smad2 or Smad3 levels at steady state, and little change in the concentrations of phosphorylated Smad2 in TGF-β-stimulated cells. We also examined transiently expressed Smad2. However, coexpression of Smurf2 and Smad2, either in the presence or absence of activated TβRI, did not alter steady-state levels of Smad2 (Fig. 2b). Furthermore, analysis of steady-state concentrations of phosphorylated and total Smad2 in transiently expressing cells revealed no significant proteasome-dependent alterations in Smad concentrations in the presence of Smurf2 and TGF-β signalling (Fig. 2c). Pulse–chase analysis also

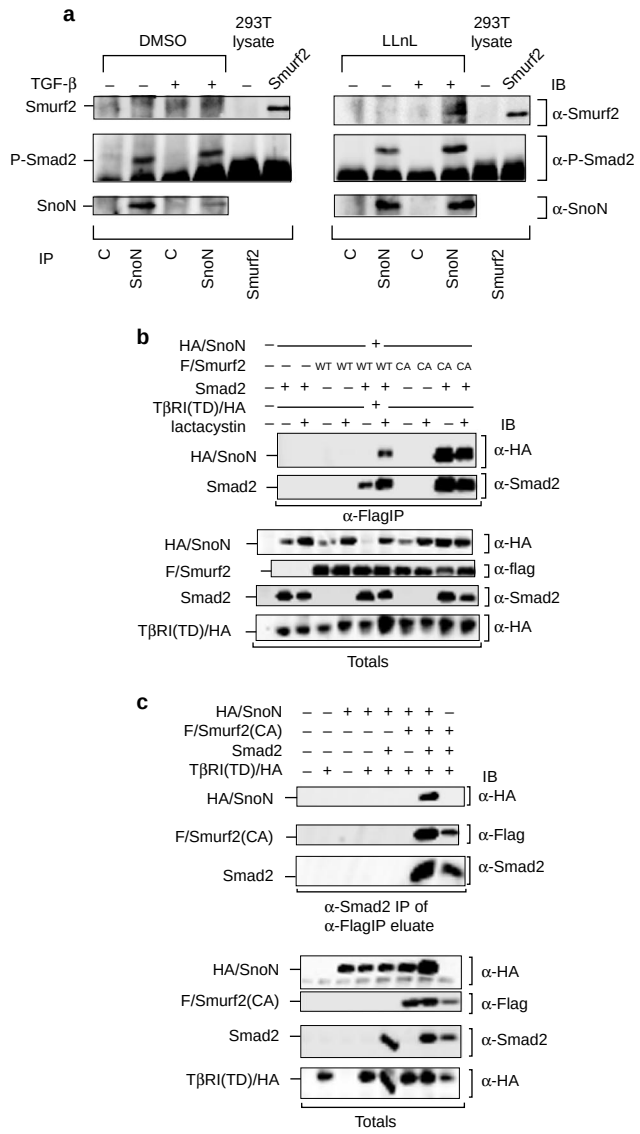

Figure 3 **Smad2 recruits Smurf2 into a complex with SnoN. a**, TGF-β induces complex formation between endogenous SnoN, Smurf2 and Smad2. U4A/Jak1 cells were pretreated for 4 h with the proteasomal inhibitor LLnL (40 μM) or a vehicle (C) and then incubated with or without TGF-β (500 pM). Cell lysates were subjected to either control (C) or SnoN immunoprecipitation and endogenous co-immunoprecipitating Smurf2 and phosphorylated Smad2 were detected by immunoblotting with anti-Smurf2 antibody (upper panels) or anti-phospho-Smad2 (α-P-Smad2) antibody (middle panel). Lysates of 293T cells transfected with Smurf2 were immunoblotted with Smurf2 antibody as a positive control. Endogenous SnoN was detected by immunoprecipitation with anti-SnoN antibodies (lower panels). **b**, 293T cells were transfected with the indicated combinations of expression vectors encoding HA/SnoN, F/Smurf2, Smad2 and constitutively active TβRI/HA. Lysates of cells, incubated in the presence or absence of lactacystin, were subjected to Smurf2 immunoprecipitation and co-immunoprecipitated proteins and detected by immunoblotting as indicated (upper two panels). Expression levels of SnoN, Smurf2, Smad2 and activated TβRI were assessed by immunoblotting (lower panels). **c**, Smurf2, Smad2 and SnoN form a trimeric complex. Flag-tagged Smurf2 was immunoprecipitated from cell lysates, eluted using Flag-tagged peptide and subjected to Smad2 immunoprecipitation. Co-immunoprecipitated SnoN was detected by immunlobloting (upper panel). Smurf2 and Smad2, present in the second immunoprecipitations, were also analysed by immunoblotting (top panel). Lower panels, total expression of SnoN, Smurf2, Smad2 and activated TβRI (total).

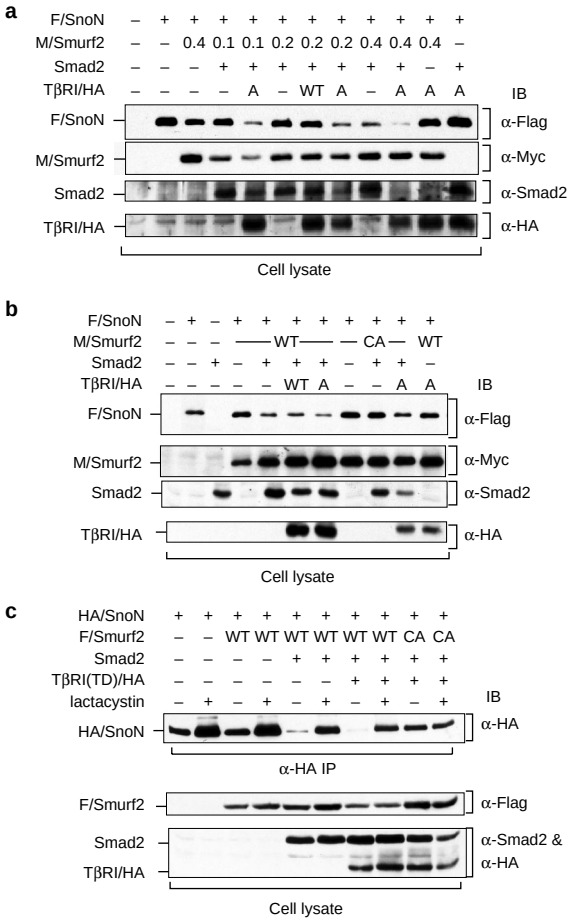

Figure 4 **Smurf2 regulates levels of SnoN at steady state. a**, Smurf2 mediates Smad2-dependent reduction of levels of SnoN at steady state. SnoN at steady state in total cell lysates (obtained from 293T cells that expressed Flag-tagged SnoN, Myc-tagged Smurf2, Smad2 and wild-type (WT) or activated (A) TβRI/HA) were determined by immunoblotting. Total levels of Smurf2, Smad2 and TβRI expression are also shown. **b**, Smurf2-dependent decrease in levels of SnoN at steady state requires the catalytic activity of the HECT domain. Levels of SnoN at steady state in the presence of wild-type Smurf2 (WT) or Smurf2(C716A) HECT-domain mutant (CA) were determined as in panel **a**. **c**, Smurf2-dependent reduction in levels of SnoN at steady state involves the proteasome pathway. Cells expressing the indicated proteins were left untreated or were treated with lactacystin, and levels of SnoN protein determined by immunoblotting (upper panel). Expression levels of the other constructs were confirmed by immunoblotting with the appropriate antibodies (lower panels).

confirmed that the turnover rate of the bulk of Smad2 was unaffected by coexpression of Smurf2 (Fig. 2d).

It has been proposed that Smurf2 can target Smad2 for degradation[30]. Our results, and those of others[31], indicate that a substantial proportion of Smad2 is not targeted by Smurf2. For both endogenous and transiently expressed Smad2, treatment with the proteasome inhibitor LLnL induced the appearance of some phosphorylated Smad2 in the absence of exogenous TGF-β signals (Fig. 2a, c). Hence it is possible that in resting cells, ubiquitin ligases such as Smurf2 may function to target this small pool of phosphorylated Smad2 to maintain the TGF-β pathway in a quiescent state. Indeed, there is some evidence that under certain conditions Smads are subject to ubiquitin-mediated proteolysis in the nucleus[34]. Nevertheless, our data indicate that upon activation of the TGF-β pathway, phosphorylated Smad2 can form a stable complex with

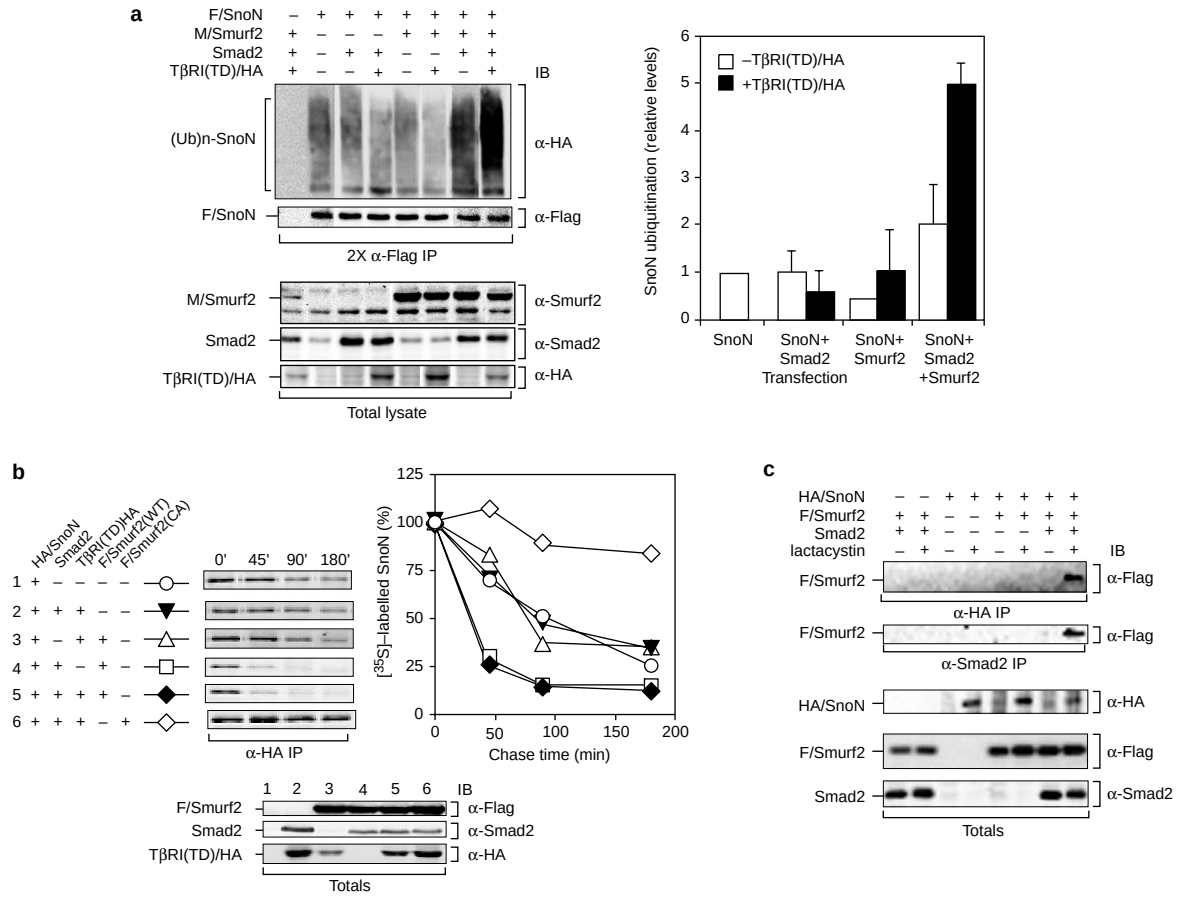

Figure 5 **Smad2–Smurf2 complex targets SnoN for ubiquitination and degradation. a**, The Smurf2/Smad2 complex enhances SnoN ubiquitination in a TGF-β-dependent manner. 293T cells were co-transfected with HA-tagged ubiquitin and various combinations of F/SnoN, M/Smurf2, untagged Smad2 and constitutively active TβRI/HA. After overnight incubation of cells with LLnL (20 μM), lysates were subjected to anti-SnoN immunoprecipitation, elution by boiling in 1% SDS, another anti-SnoN precipitation and immunoblotting (left panels). Poly-ubiquinated SnoN was quantified using a BioRad Fluor-S-Multimager, and the data were normalized to poly-ubiquitinated SnoN concentration from cells transfected with SnoN alone (right panel). The data represent the mean and s.d. of three independent experiments. The expression levels of M/Smurf2, Smad2 and activated TβRI/HA were confirmed by immunoblotting aliquots of the lysates with the appropriate antibodies as indicated. **b**, The Smurf2–Smad2-dependent increase of SnoN turnover requires the catalytic activity of Smurf2 HECT domain. 293T cells were transfected with HA/SnoN, untagged Smad2, constitutively activated TβRI/HA, and either wild-type (WT) or ubiquitin ligase mutant (CA) of Flag-tagged Smurf2. Cells were pulse-labelled with [^{35}S]-methionine for 10 min and then chased for the indicated time. The [^{35}S]-methionine labelled SnoN was immunoprecipitated and quantified by phosphorimage analysis. Results are plotted as the amount of labelled SnoN present at each time point relative to the level present at time 0. The expression of Smurf2, Smad2 and activated TβRI were confirmed by immunoblotting of the cell lysates (lower panels). **c**, Co-expression of Smad2, Smurf2 and SnoN promotes assembly of a trimeric complex in the absence of TGF-β signalling. Cells expressing the indicated proteins were incubated in the presence or absence of lactacystin. Lysates were equally divided and then subjected to SnoN or Smad2 immunoprecipitation, and amounts of co-immunoprecipitated Smurf2 were determined by immunoblotting. Lower panel, total expression level of SnoN, Smurf2 and Smad2.

Smurf2, but is not efficiently targeted for ubiquitin-mediated degradation.

Smad2 recruits Smurf2 into a complex with SnoN. The stable interaction between Smurf2 and Smad2 indicates that Smad2 might recruit substrates to Smurf2 for ubiquitin-mediated degradation. Recently, the transcriptional co-repressor SnoN was identified as a Smad-interacting protein[14–19], the proteasome-dependent degradation of which is enhanced by TGF-β signalling[17]. We therefore first considered whether endogenous Smurf2 and SnoN might associate in a TGF-β-dependent manner. To investigate this possibility, we used U4A/Jak1 cells, which express endogenous Smurf2 and in which SnoN concentration decreases in a proteasome-dependent manner in response to TGF-β signalling (Fig. 3a). In the absence of proteasome inhibitors, we were unable to detect Smurf2 in SnoN immunoprecipitates. However, the association of ubiquitin ligases with their targets usually results in rapid degradation of the substrates. Therefore, we also tested for Smurf2–SnoN interaction in the presence of LLnL. This inhibitor prevented the TGF-β-dependent reduction in SnoN concentrations and induced a concomitant enhancement in the amount of Smad2 bound to SnoN. In the presence of LLnL, endogenous Smurf2 coprecipitated with SnoN in a TGF-β-dependent manner. Thus, TGF-β induces the association of endogenous Smurf2 and SnoN.

To investigate the role of Smad2 in mediating the Smurf2–SnoN interaction, we analysed Smurf2–SnoN interactions in cells transiently expressing these proteins. In the absence of exogenous Smad2 or the proteasome inhibitor lactacystin, we detected no interaction between overexpressed Smurf2 and SnoN (Fig. 3b). However, in the presence of lactacystin, SnoN interacted with wild-type Smurf2 but only when it was co-expressed with Smad2 (Fig. 3b). Furthermore, the catalytic mutant of Smurf2, Smurf2(C716A), also bound to SnoN in a Smad2-dependent manner and this interaction was similar both

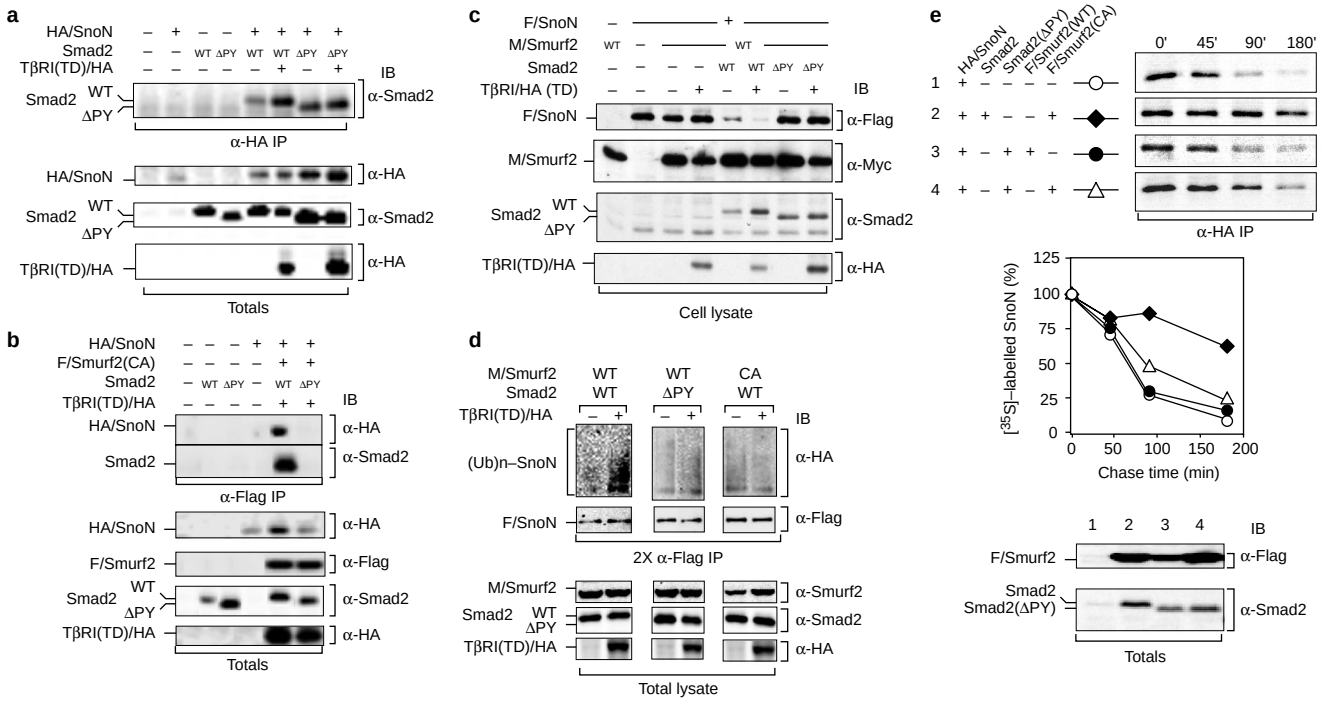

Figure 6 **The Smad2 PY motif is required to recruit Smurf2 to SnoN. a**, Smad2(ΔPY) mutant interacts normally with SnoN. Lysates of transfected cells were subjected to SnoN immunoprecipitation and to Smad2 immunoblotting. **b**, Smad2(ΔPY) does not support association of Smurf2 with SnoN. Smurf2 was immunoprecipitated from transiently transfected cells and co-immunoprecipitated SnoN and Smad2 were detected by sequential SnoN and Smad2 immunoblotting. **c**, Smad2(ΔPY) does not support the Smurf2-dependent decrease in levels of SnoN at steady state. Aliquots of total cell lysates were immunoblotted as indicated. **d**, Smad2(ΔPY) and Smurf2 (CA) do not support Smurf2/Smad2-dependent SnoN ubiquitination (determined as described in Fig. 5a). **e**, Smad2(ΔPY) can not mediate Smurf2-dependent alterations in the turnover rate of SnoN. Pulse–chase and data analysis were conducted as outlined in Fig. 5b. In **a**, **b**, **c**, **e**, total protein expression was analysed by immunoblotting with the appropriate antibodies as shown (totals).

in the presence or absence of lactacystin. These results indicate that Smad2 mediates the assembly of a trimeric complex of Smurf2, Smad2 and SnoN. To confirm this, we isolated Smurf2- and Smad2-containing complexes using a two-step procedure in which eluates from immunoprecipitates of Smurf2(C716A) were subjected to immunoprecipitation with Smad2. We then examined these precipitates for the presence of SnoN by immunoblotting. This analysis revealed that SnoN was bound to complexes of Smad2 and Smurf2 (Fig. 3c). Thus, Smad2 mediates the assembly of a trimeric complex between Smurf2, Smad2 and SnoN.

SnoN turnover is regulated by Smurf2 in a Smad2-dependent manner. We focused next on determining whether Smurf2 regulates the steady-state level of SnoN in a Smad2-dependent manner. In the absence of Smad2, low levels of Smurf2 expression had a minimal effect on the steady-state concentration of SnoN but, at higher doses, SnoN protein concentration decreased, possibly mediated by endogenous Smad2 protein (Fig. 4a, upper panel). By contrast, in the presence of both Smad2 and Smurf2, there was a decrease in the concentration of SnoN at steady state, even at the lowest levels of Smurf2 expression (Fig. 4a, upper panel). This reduction was strongly enhanced in the presence of TGF-β signalling. In the case of Smurf2(C716A), there was little change in the concentration of SnoN at steady state (Fig. 4b). Furthermore, treatment with lactacystin reversed the Smurf2–Smad2-dependent reduction in concentration of SnoN at steady state (Fig. 4c). These results indicate that Smurf2, through the catalytic activity of its HECT domain, can target SnoN for ubiquitin-proteasome mediated degradation. In these experiments, concentration of Smad2 occasionally decreased when SnoN was present. Pulse–chase analysis showed that, in the presence of SnoN, there was a slight increase in Smad2 turnover (data not shown). This may reflect a degradation of Smad2 that occurs when SnoN is targeted for ubiquitin-mediated degradation by Smurf2. This could account for the observations that Smurf2 might target Smad2, albeit with poor efficiency[30,31]. Our analysis of Smad7 also revealed that when Smurf2 is recruited to the TGF-β receptors, Smad7 turnover is enhanced[32]. Similar effects have also been noted for other E3 ubiquitin ligases[24,25]. Thus, it is possible that Smad2 is degraded within the trimeric complex.

Next we investigated whether Smurf2 alters the ubiquitination of SnoN. In the absence of proteasome inhibitors SnoN level is strongly reduced by Smad2 and Smurf2, precluding analysis of SnoN ubiquitination under these conditions. Therefore to visualize ubiquitinated species of SnoN, we inhibited the proteasome using treatment with LLnL. When expressed alone, SnoN was constitutively ubiquitinated, probably through a Smad-independent pathway, as described elsewhere[18]. Expression of either Smad2 or Smurf2 alone had little effect on ubiquitinated SnoN, however co-expression of both proteins caused a fivefold increase in ubiquitination of SnoN upon activation of TGF-β signalling (Fig. 5a).

To confirm that the alterations in levels of SnoN at steady state and ubiquitination reflect an enhanced turnover of SnoN, we monitored the rate of degradation of SnoN by pulse–chase analysis (Fig. 5b). SnoN, when expressed alone or together with the activated TGF-β receptor and either Smad2 or Smurf2, had a half-life of about 80 min. However, in the presence of Smad2 and Smurf2 the half-life of SnoN decreased to less than 40 min, either in the presence or absence of TGF-β signalling. In contrast, under the same conditions, Smurf2(C716A) increased SnoN half-life to more than 200 min. Together, these results show that in the presence of Smad2, wild-type Smurf2 enhances the turnover of SnoN, whereas

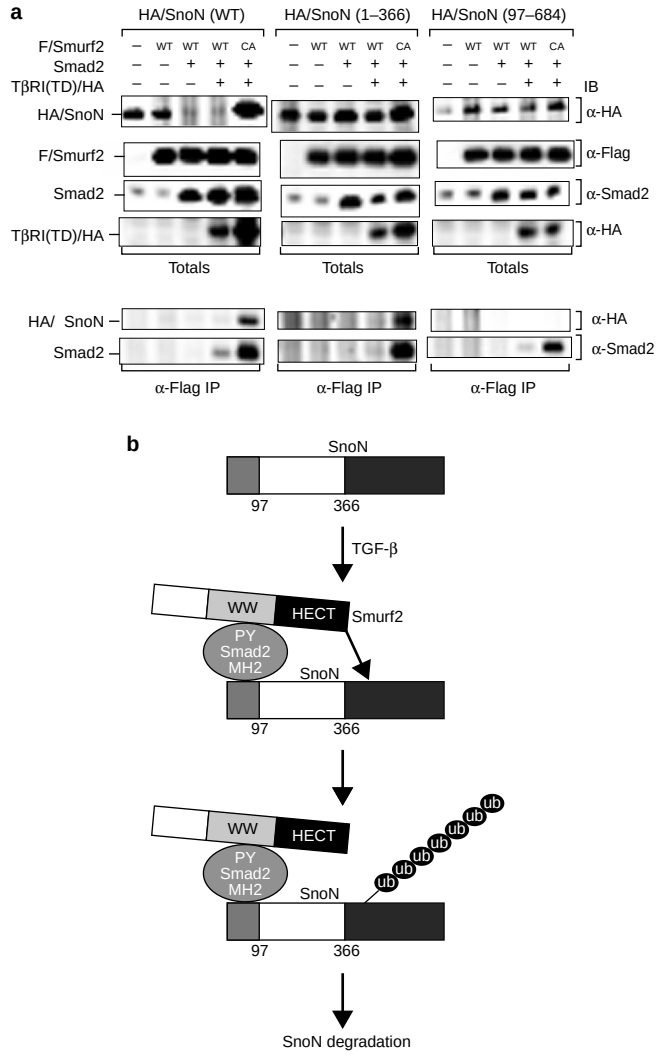

Figure 7 **Determinants on SnoN required for Smad2–Smurf2-dependent turnover and model depicting ubiquitin-mediated degradation of SnoN. a**, Determinants on SnoN required for Smad2–Smurf2-dependent turnover. 293T cells were transfected with the indicated Smurf2 constructs together with either wild-type SnoN or mutants of SnoN lacking the first 96-amino acids (SnoN(97-684)) or the last 318-amino acids (SnoN(1-366)). Lysates were divided equally and either subjected to SnoN immunoprecipitation and immunoblotting (upper panels), or Smurf2 was immunoprecipitated and co-precipitating SnoN and Smad2 assessed by immunoblotting (lower panels). Total Smurf2, Smad2 and TβRI expression was confirmed by immunoblotting with the appropriate antibodies. **b**, A model depicting ubiquitin-mediated degradation of SnoN through a TGF-β-dependent pathway that involves Smad2-dependent recruitment of Smurf2 to SnoN.

the ubiquitin ligase mutant of Smurf2 strongly reduces SnoN degradation.

Our analysis of SnoN stability indicated that Smad2 mediates Smurf2-dependent turnover even in the absence of TGF-β signalling. In contrast, our biochemical data indicated that the interaction between Smad2 and Smurf2 was TGF-β-signalling dependent. To resolve this discrepancy, we considered whether overexpression of all three components of the Smurf2–Smad2–SnoN complex might allow assembly of a trimeric complex that could target SnoN for degradation, even in the absence of signalling. To address this, we scaled up our analysis and investigated Smurf2 interactions with SnoN and Smad2 in the absence of receptor activation (Fig. 5c). To protect against proteasome-induced degradation of SnoN, cells were incubated with lactacystin. As we have shown, co-expression of Smurf2 and Smad2 did not lead to a detectable interaction, either in the presence or absence of treatment with lactacystin. Likewise, there was no interaction between SnoN and Smurf2 when they were co-expressed alone. However, in the presence of Smad2, Smurf2 associated with SnoN, and this interaction was detected only when cells were treated with lactacystin (Fig. 5c). Furthermore, in the presence of SnoN, Smurf2 interacted with Smad2. These results indicate that when all three components are overexpressed, a Smurf2–Smad2–SnoN complex can assemble in the absence of TGF-β signalling, thus allowing targeting of SnoN by Smurf2 ubiquitin ligase.

The PY motif of Smad2 is necessary for Smurf2-dependent ubiquitination and degradation of SnoN. To determine the role of Smad2 in nucleating the assembly of a Smurf2–Smad2–SnoN complex, we examined the interaction of Smad2(ΔPY) with SnoN. Smad2(ΔPY) bound SnoN normally (Fig. 6a), however, it was unable to recruit Smurf2 to SnoN (Fig. 6b). Next, we investigated the effect of Smad2(ΔPY) on the Smurf2-dependent decrease in levels of SnoN at steady state (Fig. 6c). In contrast to wild-type Smad2, Smad2(ΔPY) was no longer able to mediate a Smurf2-dependent reduction in levels of SnoN at steady state. Furthermore, Smad2(ΔPY) was unable to support Smurf2-dependent ubiquitination of SnoN (Fig. 6d). Similarly, the catalytic mutant of Smurf2, Smurf2(C716A), was unable to induce SnoN ubiquitination. Pulse–chase studies of SnoN turnover further confirmed that in the presence of Smad2(ΔPY), neither wild-type Smurf2 nor Smurf2(C716A) altered SnoN half-life (Fig. 6e). Together, these data show that the association of Smad2 with Smurf2 is mediated by the PY motif and that this interaction is necessary for recruitment of Smurf2 to SnoN.

Determinants on SnoN that are required for degradation by Smad2–Smurf2 E3 ligase. We next characterized the determinants on SnoN that are necessary for its targeting by Smad2–Smurf2 complex. For this, we used two characterized deletion mutants of SnoN. The first mutant, SnoN(1–366) contains the Smad2/3 binding site, but lacks the C-terminal end and is not degraded in response to Smad binding[16]. The second mutant, SnoN(97–684) lacks the N-terminal Smad-binding region of SnoN and thus does not interact with Smad2 (ref. 16). In contrast to wild-type SnoN, the steady-state concentration of SnoN(1–366) was unaffected by co-expression with Smurf2 and Smad2, even though this mutant interacted efficiently with Smurf2 in the presence of Smad2 (Fig. 7a). This indicates that the C-terminal region of SnoN is targeted for ubiquitination by the Smurf2-HECT domain. Consistent with this, the lysine residues that mediate TGF-β and Smad-dependent turnover of SnoN have been mapped to this region, and mutation of these sites also blocks Smad2–Smurf2-dependent turnover of SnoN and enhances the antagonism of TGF-β signalling by ectopically expressed SnoN (S.S., S.B., J.L.W. and K.L., unpublished results). In the case of SnoN(97–684), Smad2 was unable to recruit Smurf2 to the protein (Fig. 7a), which is consistent with the inability of this mutant to bind to Smad2 (ref.16; data not shown).

On the basis of these results, we propose a model (Fig. 7b) in which SnoN turnover is regulated by TGF-β stimulation through Smad2–Smurf2 ubiquitin ligase complex. According to this model, Smad2 can interact through its MH2 domain with the N-terminal region of SnoN, and at the same time can associate through its PY motif with the WW domains of Smurf2. By this mechanism, Smad2 recruits Smurf2 to SnoN, thereby allowing the HECT domain to target the C-terminal region of SnoN for ubiquitination and subsequent degradation by proteasomes.

Discussion

Ubiquitination is essential for regulating the degradation of components in signalling pathways. Smurf1, which is closely related to

Smurf2, can bind directly to BMP-regulated Smads1 and 5 and target them for degradation[29]. Smurf2 may also target R-Smads for degradation[30,31], although its activity on Smad2 is limited (ref. 31and this study). Our results however, show that R-Smad2 can function to recruit Smurf2 to SnoN, thus allowing Smurf2 to catalyse ubiquitin-mediated proteolysis of SnoN. This defines a new role for Smad2 as a component of a ubiquitin ligase complex that mediates the degradation of specific proteins in response to signalling by TGF-β.

We have shown that Smurf2 binds constitutively to Smad7 and is recruited to active TGF-β receptors where it can induce ubiquitin-dependent degradation of the receptor complex and Smad7 (ref. 32). In this case, interaction of Smurf2–Smad7 does not depend on TGF-β signalling. In contrast, the interaction between Smad2 and Smurf2 is strictly dependent on phosphorylation of R-Smad at the C-terminal SSXS motif. It is unclear which structural determinants control this phosphorylation-dependent association. One possibility is that the PY motif could be masked in unactivated R-Smad through putative autoinhibitory mechanisms. Alternatively, R-Smads exist as monomers in an unactivated state, and phosphorylation induces their assembly into higher order complexes. This may bring together multiple PY motifs that could exhibit altered avidity for the multiple WW domains of Smurf2, and thus drive phosphorylation-dependent association. This ligand-dependent association between Smurf2 and Smad2 might be extended to other PY-containing R-Smads, as we have shown for Smad3. Indeed, our preliminary data show that BMP signalling can induce an interaction between Smad1 and Smurf2 (S.B. and J.L.W., unpublished data). Thus, Smurf2 may function as a general partner for Smads. Moreover, the use of multiple Smad adaptors probably serves to increase the range of Smurf2 substrates, as for each Smad, a distinct set of interacting proteins might be recruited to Smurf2 for ubiquitin-mediated degradation.

A number of nuclear proteins that functions as R-Smad partners have been defined[4–6,12], and these interacting proteins could potentially be targeted by Smurf2 for ubiquitin-mediated degradation. One such interacting protein is SnoN. In SnoN, two determinants are required for degradation. The first determinant is the Smad interaction motif found in the first 96-amino acids of the protein, and SnoN mutants lacking this region are resistant to Smad2–Smurf2-mediated degradation. The second determinant, found in the C-terminal region, is essential for Smurf2-dependent degradation of SnoN, but it is not required for the assembly of the trimeric Smurf2–Smad2–SnoN complex. During these studies, we also examined the fork-head protein FAST, which is a Smad2 DNA-binding partner. Although Smad2 can recruit Smurf2 to FAST, there was no alteration in levels of FAST protein (S. B. and J. L. W., unpublished data). Thus, the requirement for a bipartite signal in Smad2–Smurf2 targets is probably important to insure that not all Smad2 partners are targeted for degradation in response to TGF-β signalling.

The Smad2–Smurf2 ubiquitin ligase pathway may mediate a variety of cellular responses to TGF-β that are distinct from the traditional role defined for Smads as transcriptional co-modulators. How TGF-β-dependent degradation of Smad partners, such as SnoN, functions in transmission of TGF-β signals is under investigation. Overexpression of SnoN can lead to repression of TGF-β signals, presumably through recruitment of HDAC to Smad proteins[16]. Our attempts to investigate Smurf2-dependent blockage of this activity in overexpression studies has proven difficult because Smurf2 also functions together with Smad7 to inhibit TGF-β signalling through degradation of TGF-β receptor complexes. These observations indicate that Smurf2 is essential in both the initiation and down-regulation of the pathway. Consequently, during initiation of TGF-β signalling, Smurf2 may partner with phosphorylated Smad2 to facilitate activation of the pathway by degrading inhibitors such as SnoN. Thereafter, as levels of Smad7 protein rise in response to TGF-β signalling, Smurf2 may bind to Smad7 to target TGF-β receptors for degradation, thereby shutting down the signalling pathway. Thus, Smurf2 may fulfil temporally distinct functions in TGF-β signalling through partnership with different classes of Smad proteins.

The regulated degradation of proteins in response to extracellular signals is an essential process in a number of signalling pathways. During activation of the transcription factor NF-κB, phosphorylation of the inhibitor IκB induces degradation by SCF-βTrCP ubiquitin ligase complex to release NF-κB for activation of target genes[35–37]. This ubiquitin ligase complex can also constitutively target the transcriptional activator β-catenin and stimulation of Wnt signalling inhibits this pathway to activate transcription[36,38,39]. Our finding that Smad2 can function as a TGF-β-dependent adaptor for Smurf2 provides a mechanism whereby regulated ubiquitination may function in signalling by the TGF-β family. It remains to define how ubiquitination of specific Smad partners contributes to the diverse biology processes that are associated with TGF-β and BMP signalling. □

Methods

Construction of expression vectors.

The cloning of human Smurf2 and construction of the catalytically inactive Smurf2 (Smurf2C716A) has been described[32]. Smad2(ΔPY) was generated by deleting the amino acids 221–225 by a PCR based approach.

Mammalian cell lines and transfections.

MvILu cells were maintained in minimum essential media (MEM) containing non-essential amino acids and 10% fetal calf serum (FCS). 293T and U4A/Jak1 cells were grown in Dulbecco's modified media (DMEM) supplemented with 10% FCS. 293T cells were transiently transfected using the calcium-phosphate precipitation method.

Immunoprecipitations and immunoblotting.

Immunoprecipitations and immunoblotting were performed as described[32] using anti-Flag-tagged M2 (Sigma), anti-Smad2/3 (rabbit, UBI; mouse, Transduction Labs; goat (N19), Santa Cruz), anti-phosphospecific Smad2 (UBI), anti-HA (rabbit, Santa Cruz; mouse, 12CA5 or 11.1 (Babco)), and anti-Smurf2 or antiSnoN (rabbit, H-317 (Santa Cruz)) antibodies[32]. Immunoblots were detected using the HRP-conjugated secondary antibodies and enhanced by chemiluminescence (Amersham). To show the existence of Smurf2–Smad2–SnoN trimeric complex, Flag-tagged immunoprecipitates were eluted twice using the Flag-tagged peptide (0.5 μg ml^{-1}; Sigma), and the eluates were pooled and subjected to mouse anti-Smad2 antibody immunoprecipitation. Ubiquitination assays were conducted as described[32].

Pulse chase analysis.

293T cells were transfected in 100-mm tissue culture dishes as indicated and, on the next day, were re-plated into poly-D-lysine coated 6-well plates. On the third day, cells were labelled for 10 min at 37 °C with 50 μCi ml^{-1} of [^{35}S]-methionine in methionine-free DMEM. Cell layers were then washed once and incubated in DMEM plus 10% FCS for the indicated time. At each time point of the chase, cell lysates were immunoprecipitated with anti-Smad2 polyclonal or anti-HA 12CA5 monoclonal antibody, to purify Smad2 and HA-tagged SnoN, respectively. Immune complexes were resolved by SDS–PAGE and visualized by autoradiography. A PhosphorImager (Molecular Dynamics) was used to quantify metabolically labelled Smad2 or SnoN present at each time point.

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